Horizontal and Vertical Transfer Of Oral Microbial Dysbiosis and Periodontal Disease

M.A. Payne, A. Hashim, A. Alsam, S. Joseph, J. Aduse-Opoku, W. Wade, and M.A. Curtis

Appendix

Appendix Materials and Methods

Immunohistochemistry staining

Immunocytochemistry was performed as in Zenobia et al (2013). Tissues were deparaffinized in xylene and rehydrated using decreased graded dilutions of ethanol. Tissue specimens were blocked by incubation in 1.5% H_2O_2 in methanol solution for 30 min. Primary antibody neutrophil elastase (Santa Cruz Biotechnology) was used with Vectastain ABC kit anti-rabbit. The sections were counterstained using hematoxyline. Slides were developed using DAB Peroxidase (HRP) Substrate Kit (Vector Laboratories). Sections were then de-hydrated through a dilution of alcohols and mounted.

*Next generation sequencing using Roche 454 GS-FLX+ Titanium Pyrosequencer*

Whole genomic DNA was extracted from the above swabs using the GenElute Bacterial DNA Kit (Sigma-Aldrich). The extracted samples were used as templates in PCR reactions performed in Extensor Long PCR Reddymix MasterMix (Thermo Scientific) using primer 27FYM, extended at the 5'-end to include a 30-base 454 adaptor sequence A and a unique 12 nucleotide Golay barcode for each sample, and primer 519R which has a 5'- extension with a 30-base 454 adaptor sequence B (Kistler et al. 2013). The amplified PCR products were cleaned with the Qiaquick Kit (Qiagen). When necessary, primer dimers were removed with NucleoSpin Gel and PCR Clean-up (Fisher Scientific). The amplicons were pooled in equimolar amounts prior to emulsion PCR, and unidirectionally sequenced using the Lib L v2 kit (Roche) on a Roche 454 GS-FLX+ Titanium sequencer. De-noising, de-multiplexing, trimming, chimera check, classification, clustering, Operational Taxonomic Units (OTUs) assignments, and further analyses were performed using the mothur pipeline (Schloss et al. 2009).

Statistical Analyses

Bone levels between different experimental groups were compared using one-way analysis of variance (ANOVA) and unpaired Student's t-test between 2 comparison groups. Significance was expressed at the $p < 0.05$ level (Hajishengallis et al., 2011). The statistical analyses and graphical visualization of the bone levels were performed using Graphpad Prism 7 (Graphpad Software Inc.). The differences in the composition of the overall microbiome between the treatment and control groups at each time point in each experiment were assessed by PERMANOVA analyses using the Adonis function in the Vegan package in R (Dixon P 2003). Differences in the number of CFUs of individual bacterial genera between treatment and control groups were assessed by Welch's t-test using Graphpad Prism 7.

Appendix References

Zenobia C, Luo XL, Hashim A, Abe T, Jin L, Chang Y, Jin ZC, Sun JX, Hajishengallis G, Curtis MA, Darveau RP. 2013. Commensal bacteria-dependent select expression of CXCL 2 contributes to periodontal tissue homeostasis. Cell Microbiol. 15(8): 1419-1426.
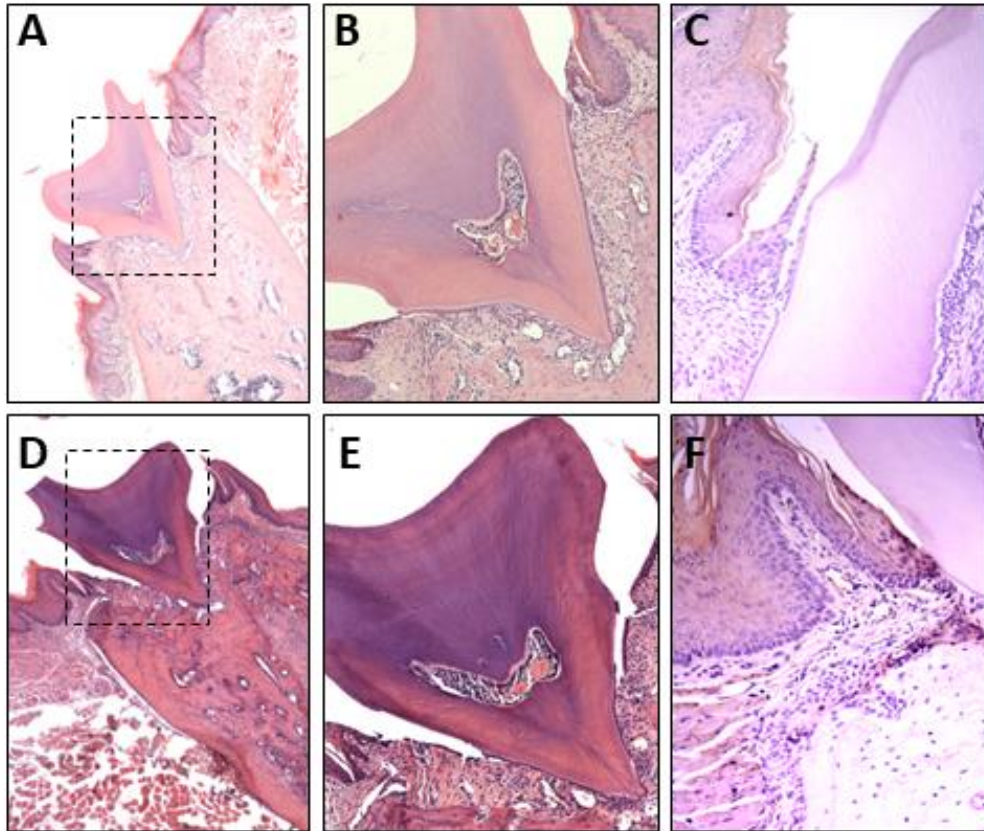
Appendix Figure 1. Comparison of gingival tissue attachment and neutrophils in SPF mice (A, B, C) and *P. gingivalis*-challenged SPF mice 6 weeks post challenge (D, E, F). Challenged mice show significant levels of loss of soft tissue attachment and higher levels of neutrophil recruitment compared to controls. Hematoxylin and eosin-stained periodontal tissue sections of SPF versus *P. gingivalis* challenged SPF mice (10x) (A & D). Boxed sections in A & D are shown at higher magnification (20x) in B and E. Immunohistochemistry of periodontal tissues of SPF and SPF challenged mice (C & F) (20x) using anti-mouse neutrophil elastase.

Appendix Table 1. Mean and standard deviation values of the observed CFUs of the individual oral bacterial communities determined by culture, of control *and P. gingivalis* treated SPF mice at 16 weeks (C/16 & Pg/16), 22 weeks (C/22 & Pg/22) and 28 weeks (C/28 & Pg28).

		Streptococcus	*Gemella*	*Staphylococcus*	*Enterobacteriaceae*	*Lactobacillus*	*Microbacterium*
C/16	**Mean**	6.28E+06	7.28E+05	1.29E+04	Not detected	Not detected	Not detected
	SD	2.31E+06	1.08E+06	8.34E+03	-	-	-
Pg/16	**Mean**	3.55E+07	6.09E+07	1.10E+07	6.26E+06	7.50E+07	3.60E+04
	SD	3.24E+07	5.76E+07	8.39E+06	1.08E+07	5.59E+07	-
	Significant?	**Not significant**	**Not significant**	**Yes, *** (p=0.0327)**	**Not significant**	**Yes, *** (p=0.0301)**	**Not significant**
C/22	**Mean**	1.23E+06	7.20E+05	1.10E+05	Not detected	Not detected	Not detected
	SD	4.42E+05	1.91E+05	1.26E+05	-	-	-
Pg/22	**Mean**	9.62E+06	6.87E+06	1.98E+06	3.27E+05	7.48E+05	2.02E+05
	SD	4.94E+06	2.89E+06	9.73E+05	4.12E+05	8.75E+05	2.67E+05
	Significant?	**Yes, ** (p=0.0074)**	**Yes, ** (p=0.0029)**	**Yes, ** (p=0.005)**	**Not significant**	**Not significant**	**Not significant**
C/28	**Mean**	6.75E+05	1.32E+06	1.09E+05	Not detected	Not detected	Not detected
	SD	3.70E+05	1.63E+05	1.20E+05	-	-	-
Pg/28	**Mean**	9.85E+06	6.57E+06	1.33E+06	9.23E+04	1.50E+07	Not detected
	SD	8.77E+06	1.83E+06	1.29E+06	1.38E+05	9.38E+06	-
	Significant?	**Not significant**	**Yes, *** (p=0.0002)**	**Not significant**	**Not significant**	**Yes, *** (p=0.011)**	**-**

The variations in the number of CFUs between test and challenged groups at each timepoint was statistically assessed using Welch's unpaired t-test (** p<0.05; *** p<0.005; **** p<0.0005).

Appendix Table 2. Mean and standard deviation values of the observed CFUs of the individual oral bacterial communities determined by culture, of control *and P. gingivalis* treated SPF mice at 6 weeks after challenge and conventionalized germ-free mice 10 weeks after co-caging.

		Streptococcus	*Gemella*	*Microbacterium*	*Staphylococcus*	*Enterobacteriaceae*	*Lactobacillus*
Control	Mean	2.35E+06	5.37E+05	Not detected	1.63E+05	Not detected	4.80E+05
	SD	2.07E+06	2.53E+05	-	1.44E+05	-	4.40E+05
Pg Challenged	Mean	1.02E+08	3.61E+07	1.39E+06	2.30E+06	5.26E+06	3.47E+07
	SD	1.47E+07	2.34E+07	1.06E+06	1.52E+06	2.56E+06	1.50E+07
Significant?		Yes, ** (p=0.006)	Not significant	Not significant	Not significant	Not significant	Yes, * (p=0.05)
Conventionalized	Mean	1.09E+08	4.38E+07	2.07E+06	4.01E+06	3.19E+06	Not detected
	SD	5.58E+07	3.03E+07	2.41E+06	3.23E+06	2.88E+06	-

The variations in the number of CFUs between test and challenged groups at each timepoint was statistically assessed using Welch's unpaired t-test (** $p<0.05$; *** $p<0.005$; **** $p<0.0005$).

Appendix Table 3. Mean and standard deviation values of the observed CFUs of the individual oral bacterial communities determined by culture, of control *and P. gingivalis* treated SPF mice at 16 weeks (C/16 & Pg/16) and litters of controls and Pg treated parents at 8 (FC/8 & FPg/8), 16 (FC/16 & FPg/16) and 28 weeks (FC/28 & FPg/28).

		Streptococcus	*Gemella*	*Staphylococcus*	*Enterobacteriaceae*	*Lactobacillus*	*Enterococcus*	*Microbacterium*
C/16	Mean	6.28E+06	7.28E+05	1.29E+04	Not detected	Not detected	Not detected	Not detected
	Std. Dev	2.31E+06	1.08E+06	8.34E+03	-	-	-	-
Pg/16	Mean	3.55E+07	6.09E+07	1.10E+07	6.26E+06	7.50E+07	Not detected	3.60E+04
	Std. Dev	3.24E+07	5.76E+07	8.39E+06	1.08E+07	5.59E+07	-	-
	Significance	Not significant	Not significant	Yes, * (p=0.0327)	Not significant	Yes, * (p=0.0301)	-	Not significant
FC/8	Mean	5.65E+06	3.55E+06	5.62E+04	Not detected	Not detected	Not detected	Not detected
	Std. Dev	2.65E+06	2.52E+06	5.10E+04	-	-	-	-
FPg/8	Mean	6.70E+07	4.72E+07	3.65E+07	6.25E+06	2.85E+07	3.04E+06	2.12E+06
	Std. Dev	2.00E+07	1.15E+07	2.93E+07	3.42E+06	1.59E+07	2.87E+06	2.86E+06
	Significance	Yes, *** (p=0.0006)	Yes, *** (p=0.0001)	Yes, * (p=0.028)	Yes, ** (p=0.0065)	Yes, ** (p=0.0071)	Yes, * (p=0.048)	Not significant
FC/16	Mean	6.09E+06	5.50E+05	9.21E+05	Not detected	Not detected	Not detected	Not detected
	Std. Dev	9.82E+05	1.77E+05	1.53E+06	-	-	-	-
FPg/16	Mean	7.31E+07	3.09E+07	6.14E+06	1.31E+06	2.91E+07	4.85E+05	Not detected
	Std. Dev	2.24E+07	2.73E+07	4.49E+06	1.14E+06	2.56E+07	8.95E+05	-
	Significance	Yes, *** (p=0.0007)	Yes, * (p=0.041)	Yes, * (p=0.036)	Yes, * (p=0.037)	Yes, * (p=0.038)	Not significant	-
FC/28	Mean	6.55E+06	4.22E+06	5.81E+05	Not detected	1.10E+04	Not detected	Not detected
	Std. Dev	3.24E+06	1.86E+06	2.36E+05	-	7.79E+03	-	-
FPg/28	Mean	8.90E+07	5.03E+07	9.29E+06	2.57E+05	2.95E+07	4.18E+05	Not detected
	Std. Dev	3.04E+07	1.54E+07	1.14E+07	2.11E+05	1.72E+07	7.41E+05	-
	Significance	Yes, ** (p=0.0011)	Yes, *** (p=0.0006)	Not significant	Yes, * (p=0.03)	Yes, ** (p=0.0084)	Not significant	-

The variations in the number of CFUs between test and challenged groups at each timepoint was statistically assessed using Welch's unpaired t-test (** $p<0.05$; *** $p<0.005$; **** $p<0.0005$).

Appendix Table 4. Mean and standard deviation values of the observed CFUs of the individual oral bacterial communities determined by culture, of control and *P. gingivalis* treated SPF mice (C & Pg), pre-antibiotics (C/T$_0$ & Pg/1), immediately post-antibiotics (C/Abs & Pg/Abs) and at 8, 16 and 22 weeks post antibiotics (C/8, C/16, C/22 & Pg/8, Pg/16, Pg/22).

		Streptococcus	*Gemella*	*Staphylococcus*	*Enterobacteriaceae*	*Lactobacillus*	*Bacteroides*	*Enterococcus*
C/T0	**Mean**	7.04E+06	6.64E+06	2.58E+06	3.33E+04	2.36E+06	Not detected	Not detected
	SD	2.21E+06	5.88E+06	2.01E+06	1.62E+04	1.62E+06	-	-
Pg/1	**Mean**	2.04E+08	5.08E+07	8.92E+06	7.90E+06	2.04E+07	1.24E+07	Not detected
	SD	7.29E+07	1.95E+07	5.11E+06	8.63E+06	2.11E+07	8.79E+06	-
	Significant?	Yes, ** (p=0.0037)	Yes, ** (p=0.0054)	Yes, * (p=0.0473)	**Not significant**	**Not significant**	**Not significant**	-
C/Abs	**Mean**	3.76E+06	Not detected	3.85E+05	Not detected	7.35E+05	Not detected	Not detected
	SD	3.60E+06	-	2.68E+05	-	6.58E+05	-	-
Pg/Abs	**Mean**	4.54E+06	Not detected	2.00E+05	2.05E+04	3.26E+05	Not detected	Not detected
	SD	3.54E+06	-	1.32E+05	3.54E+03	1.71E+05	-	-
	Significant?	**Not significant**	-	**Not significant**	**Not significant**	**Not significant**	-	-
C/8	**Mean**	9.12E+07	2.10E+05	1.37E+07	6.92E+05	7.62E+06	Not detected	Not detected
	SD	1.50E+07	2.83E+04	2.12E+07	1.24E+06	1.00E+07	-	-
Pg/8	**Mean**	9.38E+07	6.30E+06	1.83E+07	9.39E+05	2.10E+07	Not detected	Not detected
	SD	2.66E+07	-	2.13E+07	1.36E+06	2.02E+07	-	-
	Significant?	**Not significant**	**Not significant**	**Not significant**	**Not significant**	**Not significant**	-	-
C/16	**Mean**	9.32E+06	2.28E+06	4.68E+06	1.19E+05	2.56E+06	Not detected	Not detected
	SD	4.83E+06	1.52E+06	2.23E+06	7.98E+04	5.13E+05	-	-
Pg/16	**Mean**	1.02E+08	1.95E+07	3.44E+06	2.84E+06	2.31E+07	Not detected	Not detected
	SD	3.91E+07	5.07E+06	2.14E+06	2.32E+06	1.74E+07	-	-
		Yes, * (p=0.017)	Yes, ** (p=0,0047)	**Not significant**	**Not significant**	**Not significant**	-	-
C/22	**Mean**	6.74E+06	3.65E+06	3.40E+05	1.04E+05	Not detected	Not detected	Not detected
	SD	2.54E+06	2.52E+06	1.34E+05	1.57E+05	-	-	-
Pg/22	**Mean**	1.88E+08	5.00E+07	4.39E+06	1.60E+07	2.06E+07	0.00E+00	2.35E+07
	SD	7.96E+07	1.61E+07	3.96E+06	5.62E+06	1.73E+07	0.00E+00	3.86E+07
		Yes, * (p=0.05)	**Not significant**	**Not significant**	Yes, * (p=0.039)	**Not significant**	-	-

The variations in the number of CFUs between test and challenged groups at each timepoint was statistically assessed using Welch's unpaired t-test (** p<0.05; *** p<0.005; **** p<0.0005).

Appendix Table 5. Comparison of the microbial populations of each group of mice (control and *P. gingivalis* challenged) before antibiotics (T0) compared to the timepoints immediately after administration of antibiotics (Abs), followed by 8, 16 and 22 weeks after antibiotics.

	Pre-Antibiotics (T0)	Antibiotics (Abs)	Pre-Antibiotics (T0)	8 weeks post-Antibiotics (8)	Pre-Antibiotics (T0)	16 weeks post-Antibiotics (16)	Pre-Antibiotics (T0)	22 weeks post-Antibiotics (22)
Control	** (p=0.007; R^2=0.44)		** (p=0.006; R^2=0.81)		Not significant		* (p=0.033; R^2=0.27)	
P. gingivalis **Challenged**	** (p=0.008; R^2=0.75)		** (p=0.01; R^2=0.49)		Not significant		Not significant	

Statistical significance of the differences in the populations was determined by PERMANOVA analysis using the Adonis function in the Vegan package in R (** $p < 0.05$; *** $p < 0.005$; **** $p < 0.0005$).